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Biosynthesis of the vitamin K-dependent matrix Gla protein (MGP) in chondrocytes: a fetuin–MGP protein complex is assembled in vesicles shed from normal but not from osteoarthritic chondrocytes

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SUMMARY

Objective: Mineralization has been observed in osteoarthritic cartilage but the mechanisms are incompletely understood. Vitamin K is an essential cofactor in post-translational modification of proteins where specific Glu residues become modified to Ca^{++} binding γ-carboxyglutamic acid residues (Gla). One such protein, matrix Gla protein (MGP), is a known mineralization inhibitor. This study determined if synthesis of MGP and formation of a fetuin–MGP protein complex was altered in chondrocytes and vesicles from osteoarthritis (OA) cartilage.

Methods: Chondrocytes and vesicles were isolated from normal and OA human articular cartilage and lysates prepared. Specific antibodies were used in immunoblotting to detect the mature fully γ-carboxylated form of MGP (cMGP) and non-γ-carboxylated MGP (ucMGP) as well as fetuin and MGP–fetuin complexes. γ-carboxylase activity was measured by $^{14}CO_2$ incorporation into the carboxylase peptide substrate FLEEL. Immunocytochemistry was used to examine fetuin in cartilage sections and uptake of biotin-labeled fetuin by isolated chondrocytes.

Results: Chondrocytes and vesicles from osteoarthritic tissue produced significantly less cMGP compared to those from normal cartilage. This correlated with significantly less vitamin K-dependent γ-carboxylase enzyme activity in OA chondrocytes. Fetuin was found to be present in articular cartilage and cultured chondrocytes were capable of fetuin uptake. A fetuin–MGP complex was identified in normal chondrocytes and in vesicles shed from these cells but not in OA cells or vesicles.

Conclusions: The absence of cMGP and of the cMGP–fetuin complex in OA cells and OA vesicles may be an important mechanism for increased mineralization of osteoarthritic cartilage.

Introduction

Osteoarthritis (OA) is characterized by a progressive loss of articular cartilage accompanied by new bone formation and often synovial proliferation that culminates in pain, loss of joint function, and disability. During the development of OA, articular chondrocytes can assume the hypertrophic phenotype that is characteristic of the mineralizing region of the growth plate[1,2]. Chondrocyte hypertrophy in OA is commonly associated with mineralization in the cartilage matrix[3]. Crystal formation and abnormal mineralization in the articular cartilage may contribute to the progression of OA[4].

It is now well established that vitamin K is essential in prevention of soft tissue mineralization[5–7]. In the classical experiment carried out by Luo *et al.*[7], where the vitamin K-dependent protein matrix Gla protein (MGP) was deleted, mice developed severe calcification of cartilage and the arterial wall with death as an early endpoint. Vitamin K is an essential cofactor in post-translational modification of a family of proteins where specific Glu residues in the protein sequence become modified to Ca^{++} binding γ-carboxyglutamic acid residues (Gla)[8]. This modification of coagulation factors made in the liver is essential for normal hemostasis. We have shown previously that chondrocytes synthesize MGP and possess an active post-translational γ-carboxylation system[9,10]. There is also evidence that MGP binds bone morphogenetic proteins-2 and -4 (BMPs-2 and BMPs-4) which prevents these growth factors from transforming cells into bone forming cells[11–13].

Since the pathological transformation of normal chondrocytes into OA chondrocytes involves chondrocyte hypertrophy and

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matrix mineralization, it is our hypothesis that this transformation is accompanied by a loss of vitamin K function and altered biosynthesis of the vitamin K modified calcification inhibitory protein MGP. Altered MGP function could also allow for an increase in the local activity of BMPs which could promote osteophyte formation. Indeed, one epidemiologic study has shown a correlation between low plasma levels of phylloquinone (primary plasma form of vitamin K) and the presence of osteophytes on knee radiographs[14]. The objective of the present study was to determine if chondrocytes from OA cartilage, when compared to cells isolated from normal cartilage, had altered production of the mature, fully γ-carboxylated MGP.

In order to investigate the calcification inhibitory property of MGP as it works *in vivo*, it is necessary to combine such studies with studies on fetuin which can bind the mature fully-γ -carboxylated form of MGP[15]. The blood born protein α-fetuin (fetuin), also known as α_2-Hermans–Schmid Glycoprotein is synthesized in the liver and accumulates in high concentration in bone[16–18]. Fetuin has been shown to bind mature MGP but not to the un- or incompletely γ-carboxylated MGP[17]. The way the fetuin–MGP complex works as an anti-calcification inhibitory system is not completely understood but it is likely that the complex is a needed carrier of the highly insoluble protein MGP. We[19] and others[20] have shown that fetuin is taken up by vascular smooth muscle cells (VSMCs) by an unknown uptake mechanism and fetuin may have an intra-cellular function as a carrier of MGP. It has been shown that both MGP and fetuin are present in vesicles shed by VSMCs[20]. The complex may initially be packed into intra-cellular lipid vesicles as suggested by Reynolds *et al.*[20,21]. In the present study, extracellular vesicles from normal and osteoarthritic chondrocytes were isolated to search for the fetuin–MGP complex shed by the cells. As shown by our data, the complex is present in vesicles from normal but not in vesicles from OA chondrocytes which supports the hypothesis of the existence of an enhanced calcification environment in osteoarthritic lesions and suggests that vitamin K deficiency may contribute to abnormal mineralization in OA.

Materials and methods

Materials

The human cMGP and ucMGP monoclonal peptide antibodies were directed against residues 35–54 in the human Gla region of the MGP sequence[22]. The cMGP antibody is conformation specific and only recognizes fully γ-carboxylated MGP when Ca^{++} binds to the Gla residues[22,23]. The human ucMGP monoclonal peptide antibody is directed against the same epitope (residues 35–54) of the MGP sequence, though with the Glu residues in the peptide used for antibody production and not Gla residues. Thus the ucMGP antibody recognizes only non- or partially γ-carboxylated MGP (antibodies provided by Vascular Products, Maastricht, Netherlands). The N-terminal human MGP polyclonal rabbit peptide antibody (residues 23–53) was made by Alpha Diagnostic International (San Antonio, TX). The antibody made against this synthetic peptide recognizes human MGP independently of its γ-carboxylation modification[23]. The antibody was affinity purified by our laboratory as described[23]. Sepharose–protein-A/G beads were purchased from Sigma (St.Louis, MO). EZ-Link sulfosuccinimidyl 6-(biotinamido)hexanoate and biotin were from Pierce (Rockford, IL). Mouse monoclonal IgG anti-human fetuin-A (H-4): sc-133146 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Affinity purified goat anti-human fetuin polyclonal antibodies were from R&D Systems, Inc. (Minneapolis, MN). Mouse anti-biotin IgG, horseradish peroxidase-conjugated goat anti-mouse IgG, horseradish peroxidase-conjugated rabbit anti-goat IgG, and rhodamine-labeled donkey anti-mouse IgG were from Jackson Immuno Research Laboratories, Inc. (West Grove, PA).

Chondrocyte isolation and culture

Normal human ankle and knee articular cartilage was obtained from tissue donors within 48 h of death through the National Disease Research Interchange (Philadelphia, PA) or through the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL) in accordance with institutional protocol. Each donor specimen was graded for degenerative changes based on the five-point Collins scale, as modified by Muehleman *et al.*[24]. Osteoarthritic cartilage was discarded tissue obtained after knee replacement surgery for a diagnosis of OA. Cartilage was dissected from the joints and digested in a sequential manner with pronase and then overnight with collagenase as previously described[25]. Viability of isolated cells was determined using trypan blue and cells were counted using a hemocytometer. Monolayer cultures were established by plating cells in 10 cm or six-well plates at 2×10^6 cells/ml in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 medium supplemented with 10% FBS. Plates were maintained for approximately 5–7 days with feedings every 2 days until they reached 100% confluency prior to experimental use. Cells were not passaged. For experiments with cells in serum-free medium, the attached cells were washed with phosphate buffered saline (PBS) before the DMEM/F-12 medium without serum was added. Depending on the experimental plan, cells were cultured for 24 or 48 h in serum-free medium. Fresh serum-free medium was added after 24 h.

Preparation of vesicles from normal and osteoarthritic chondrocytes

The procedure used for isolation of chondrocyte shed vesicles was adapted from the work published by Reynolds *et al.*[21] in their effort to isolate vesicles from cultured VSMCs. Collagenase treatment of the cultures was not carried out prior to removing the cells from the dishes. Confluent chondrocytes cultured either for 24 or 48 h in serum-free medium were washed twice with PBS and removed from the 10 cm culture dish with a cell scraper. The cell suspension was centrifuged at 2500 rpm in an Eppendorf Centrifuge 5810R centrifuge in order to remove cells and cell debris. The supernatant was carefully removed and subjected to centrifugation at 100,000×*g* for 45 min in a Beckman L8-M Ultracentrifuge at 4°C. The pelleted vesicles were stored frozen at −85°C until used for the experiments.

Electron microscopy

Vesicles were resuspended in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer pH 7.3. The sample was post fixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h. Subsequently, the sample was dehydrated and embedded in Spurr's resin and cured overnight in a 60°C. Eighty nm sections were cut with an ultramicrotome, stained with uranyl acetate and lead citrate, and viewed with a Philips 400 TEM.

Alkaline phosphatase activity measurements

Vesicles were resuspended in PBS and washed two times with PBS followed by re-pelleting by centrifugation at 100,000×*g*. The washed pellets were lysed on ice for 30 min in 100 μl of 0.1 M glycine buffer, pH 9.6 containing 1% NP-40, 1 mM $MgCl_2$, 1 mM $ZnCl_2$. After lysis, the samples and 100 μl of the alkaline phosphatase substrate solution included in the alkaline phosphatase kit (Bio-Rad, Herculus, CA) were added to wells on microtiter plates and incubated for 15 min at 37°C. After incubation, the reaction was stopped by adding NaOH and the optical density read at 405 nM to

determine release of the yellow product p-nitrophenolate. Specific enzyme activity was measured as nmol product produced per min and mg of protein.

Immunoprecipitation, one-dimensional-SDS-PAGE, and Western blotting

Cells were lysed on ice for 30 min in 50 mM Tris, 1% Nonidet P-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2, containing 10 μg/ml of the Sigma protease inhibitor mixture for use with mammalian cell and tissue extracts (RIPA buffer). After lysis, cell debris was removed by centrifugation at 12,000×*g* for 30 min, and the supernatant was collected. Prior to immunoprecipitation, the supernatant was precleared by absorbing it with Sepharose–protein-A/G beads for 1 h at 4°C. The beads were removed by centrifugation, and the supernatant was immunoprecipitated by adding either mouse monoclonal anti-human fetuin antibody or affinity purified goat anti-human fetuin antibody. For control experiments, preimmune mouse or goat IgGs were added to the respective samples. The antibody mixtures were allowed to react on a rotating devise overnight at 4°C. Then, Sepharose–protein-A/G beads were added, and the mixture was incubated for an additional 2 h at 4°C. The beads were washed four times in cold lysis buffer. For one-dimensional-SDS-PAGE, immune complexes were released from the beads by boiling them in SDS-PAGE buffer containing 5% mercaptoethanol (ME). Samples were loaded onto 8–16% CRITERION SDS-PAGE gels (Bio-Rad, Richmond, CA), and electrophoresed proteins transferred to polyvinylidene fluoride (PVDF) membranes for Western blotting. Total protein in isolated vesicles from normal and OA chondrocytes, when separated by one-dimensional-SDS-PAGE, were stained with a silver staining kit purchased from Bio-Rad (Richmond, CA).

Two-dimensional (2-D)-SDS-PAGE

Cell proteins in RIPA buffer were precipitated with ice-cold acetone and washed with trichloroacetic acid and ether/ethanol. The final dried protein precipitate was dissolved in the 8 M urea containing isoelectric focusing (IEF) sample buffer 1 obtained from Bio-Rad (Richmond, CA). Prior to IEF, insoluble material was removed by centrifugation, and protein samples were absorbed onto 11 cm agarose/plastic strips with immobilized ampholyte gradients from pH 3 to 10 (Bio-Rad). IEF of the proteins was carried out in a Bio-Rad PROTEAN II IEF apparatus according to the supplier's instructions and separated in the second dimension on 8–16% SDS CRITERION gels. Transfer of the 2-D-SDS-PAGE separated proteins to PVDF membranes for Western blotting was carried out as described previously our laboratory[19]. For staining of the proteins on the PVDF membrane following Western blotting of the 2-D blots, the membranes were washed briefly in distilled water and stained for 2 min in 50% ethanol, 10% acetic acid containing 0.2% Coomassie Brilliant blue. When the stained membranes were dried, the proteins appeared as blue spots against a white background. It was found that this staining procedure could only be carried out if the PVDF membrane had been blocked with fat-free milk powder.

Partial purification of human bone MGP

Small pieces of human bone left over after removal of the cartilage were soaked in liquid nitrogen and crushed into a fine powder with a strong mortar. The powder was suspended in 10% trifluroacetic acid overnight at 4°C and the unsolubilized bone removed by filtration and centrifugation. The supernatant was dialyzed extensively against distilled water for 2 days and a precipitate of protein sediment at the bottom of the dialysis bag. The sediment was harvested and lyophilized and stored at −20°C for use as a crude mixture of bone matrix proteins enriched in mature MGP.

γ-Carboxylase activity

γ-Carboxylase activity was assayed in 24 h cultured chondrocytes from normal ($n=2$) and osteoarthritic tissues ($n=2$) as $^{14}CO_2$ incorporation into the synthetic γ-carboxylase peptide substrate FLEEL as described[9]. The reaction was triggered by adding chemically reduced vitamin K_1H_2 (100 μg/ml) to the assay mixture. Pairs of normal and OA samples were tested at the same time and each sample was measured in triplicate. The results were analyzed by Student's *t*-test using StatView software (SAS Institute, Cary, NC).

Biotin labeling of fetuin

Five mg of α_2-hs-Glycoprotein (fetuin) isolated from human plasma (Sigma, St.Louis MO, #G0S16) was dissolved in 0.5 ml of PBS. A 20-fold molar excess of EZ-Link sulfosuccinimidobiotin was added to the fetuin solution, and the mixture reacted for 30 min at room temperature in the dark on a rotating shaker. The reaction mixture was gel-filtrated on Sephadex G-25 in PBS, and the void volume fraction containing biotin-conjugated fetuin was collected. The biotin-conjugated fetuin was dialyzed against PBS overnight at 4°C and sterile-filtrated before added to cell culture media.

Immunohistochemistry

Normal chondrocytes cultured in serum-free DMEM/F-12 medium were depleted of endogenous fetuin by incubating the cells for 15 min at 37°C in the serum-free medium as described by Lorenzo *et al.*[26]. Fetuin binding to the chondrocyte membrane was investigated by incubating depleted cells with biotin-labeled fetuin (50 μg/ml) in the serum-free medium for 30 min at 4°C[26]. Cells were washed with ice-cold PBS and fixed for 10 min in 4% paraformaldehyde. For uptake studies, cells incubated for 30 min at 4°C in the presence of 5 μg/ml biotin-labeled fetuin were exposed to serum-free medium at 37°C for an additional 30 min before being fixed in paraformaldehyde[26]. For fluorescent staining, fixed cells were incubated at room temperature for 30 min with biotin-specific monoclonal antibodies (1:100 dilution), followed by incubation with rhodamine-labeled donkey anti-mouse IgG (1:200 dilution) for 30 min at room temperature. Rhodamine epifluorescence images were obtained using a Zeiss Axioskop equipped with a digital camera and Axovision imaging software. Final images were processed using Adobe Photoshop Version 7.0.1. Images of rhodamine epifluorescence were also obtained by confocal microscopy. A Zeiss Model 510 laser scanning confocal microscope equipped with a 40× water immersion objective was used. The optical slice thickness was 2.1 μm for each image. Cryo-embedded cartilage was purchased from the National Disease Research Interchange. Microtome cut sections were rehydrated in PBS and non-specific immuno-globulin binding sites blocked by treatment for 20 min with 5% donkey serum in PBS with 0.5% Triton X-100. For identification of fetuin in chondrocytes in their lacunas, an anti-human fetuin monoclonal antibody was used as the first antibody diluted in PBS/TX-100. Secondary antibodies were applied for 1 h. The secondary antibody was rhodamine red X-conjugated donkey anti-mouse IgG. Confocal images were again collected with the Zeiss confocal laser scanning microscope (LSM 510).

Mass spectrometry

Identification of MGP in the cell culture medium from normal chondrocytes was carried out by the proteomic core laboratory at

Virginia Bioinformatics Institute, Blacksburg, VA. MS/MS analysis of proteins in the vesicle pellet isolated from normal chondrocytes identified MGP among the proteins present in the pellet. Identification was made on 100% sequence match with two unique peptides (23% coverage) and a 1% false discovery rate.

Results

Carboxylation status of MGP produced by normal and osteoarthritic chondrocytes

Extracts from both normal chondrocytes and OA chondrocytes cultured for 24 h in serum-free medium contained MGP recognized by the N-terminal MGP antibody that recognizes MGP independent of its carboxylation status [Fig. 1(A)]. Two immunoreactive bands were noted in these blots where the upper band was at the same position as partially purified human MGP extracted from human bone. When the chondrocyte extracts were immunoblotted with the cMGP antibody the upper band present in normal chondrocyte extracts was the predominant band with a much fainter band detected in OA chondrocytes [Fig. 1(B)]. The lower band, which was predominant in the OA extracts, reacted with the monoclonal ucMGP antibody [Fig. 1(C)]. This separation of the mature cMGP form and the inactive ucMGP was accomplished by separation of the two forms of the proteins in an 8–16% CRITERION gradient gel. A difference in the apparent molecular masses of the cMGP and the ucMGP, when run on SDS-PAGE gels, has been observed earlier[27]. From these experiments, we could conclude that both normal and OA chondrocytes synthesize approximately the same amount of total MGP protein (compare lanes NC and OA in panel A) but synthesis of the mature MGP was significantly reduced in the OA cells. A faint band reacting with the anti-ucMGP antibody was seen also in the extract from the normal cells (panel C, lane NC) and most likely represents newly synthesized precursors of MGP that have not yet been γ-carboxylated. To gain insight into the process of γ-carboxylation of proteins in normal and OA chondrocytes, we tested γ-carboxylase activity in both cell types cultured for 24 h in serum-free medium. Specific γ-carboxylase activity in normal chondrocytes was some five-times higher than the activity measured in OA chondrocytes. Normal chondrocytes had an activity of 5010 ± 13.4 cpm/mg protein (mean ± s.d.) compared to OA chondrocytes at 1033 ± 47.4 (mean difference 3977, 95% confidence interval (CI) 3827–4127, $P < 0.0001$). These data support the Western blotting data demonstrating deficient production of cMGP in OA chondrocytes.

Ability of normal but not OA chondrocytes to produce an MGP–fetuin complex

MGP is a highly insoluble protein, which, during its biosynthesis, follows the secretory pathway from the ER to the Golgi apparatus to acquire its post-translational modifications which include γ-carboxylation and phosphorylation. As noted in the introduction, binding of MGP to fetuin may facilitate its transport and/or exit from the cell. We confirmed the presence of fetuin in normal chondrocyte extracts by its characteristic shape and location on a Coomassie blue stained membrane [Fig. 2(A)] and its reaction with an anti-fetuin antibody [Fig. 2(B)]. To find out if a cMGP–fetuin complex exists in chondrocytes, we immunoprecipitated RIPA buffer extracts of normal chondrocytes using a fetuin monoclonal antibody and separated the proteins by 2-D-SDS-PAGE. Western blotting with the anti-cMGP antibody revealed the characteristic shape and location of the fetuin molecule[19], as it shows up in 2-D-SDS-PAGE gels [Fig. 2(C)]. The anti-fetuin immunoprecipitant also contained cMGP and a non-specific reaction of the secondary horseradish conjugated goat anti-mouse IgG with the heavy chain of the mouse anti-fetuin IgG used to precipitate the fetuin–cMGP complex. Together these experiments provide strong evidence for the existence of an intra-cellular fetuin–cMGP complex. Consistent with data obtained previously[19], no findings of immune reactions with the fetuin protein present in 2-D-SDS-PAGE gels were seen when Western blotted with the anti-ucMGP antibody (data not shown).

Having demonstrated that a fetuin–cMGP complex is formed intra-cellularly in normal chondrocytes, we next searched for the complex in vesicles shed from either normal or OA chondrocytes. We selected to use a vesicle isolation procedure which does not include collagenase treatment to release vesicles from the cell culture in order to examine the spontaneous production of vesicles rather than those generated by collagenase. For this, we used a modification of the procedure described by Reynolds *et al.*[21] in their effort to isolate matrix vesicles from VSMCs (see Materials and methods). We analyzed the vesicles shed from OA chondrocytes and found a mixture of sizes ranging from about 75 nM to 200 nM [Fig. 3(A)]. The vesicles had the size and appearance of what previous studies have called matrix vesicles[21,28,29]. We tested a preparation of the vesicles for alkaline phosphatase activity and measured a specific activity of 246 ± 14 nmol/min/mg of protein. This level of activity was almost two-fold higher than the 125 ± 14 nmol/min/mg of protein reported using a similar assay with matrix vesicles isolated from control chick growth plate chondrocyte cultures reported by Kirsch *et al.*[29] but much lower than that reported for mineralizing matrix vesicles induced by vitamin C/phosphate treated growth plate chondrocytes in the same study. Because we did not test the calcification potential of the vesicles, we refer to the particles isolated in the present study as vesicles rather than authentic matrix vesicles. We identified MGP in the vesicle fraction shed from normal chondrocytes by mass spectrometry (see Materials and methods). The vesicles were further analyzed by SDS-PAGE and silver staining which detected a number of protein bands [Fig. 3(B)] in vesicles from normal and OA cultures. Western blotting with the anti-cMGP antibody only recognized cMGP in vesicles from normal chondrocytes [Fig. 3(C)] while Western blotting for fetuin recognized fetuin in both normal and OA vesicles [Fig. 3(D)]. The fetuin content appeared to be lower in vesicles harvested after 48 h compared to those cultured for only

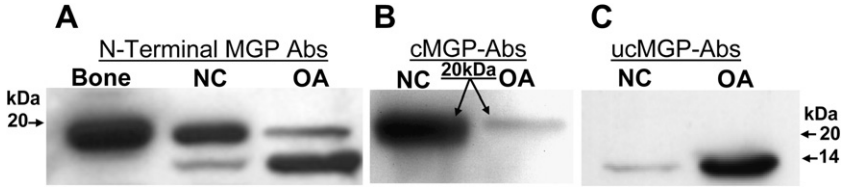

Fig. 1. Production of MGP by normal and OA chondrocytes. Proteins in RIPA buffer extracts from normal (NC) and osteoarthritic (OA) chondrocytes were Western blotted with (A) a polyclonal N-terminal human MGP peptide antibody which recognizes human MGP independently of its γ-carboxylation status, (B) conformational specific cMGP peptide antibody which recognizes the mature fully γ-carboxylated form of MGP in the presence of Ca^{++}, and (C) the ucMGP antibody which recognizes none or under-γ-carboxylated human MGP. Equal amounts of total protein were added in each lane. Lane Bone in A contains human MGP protein partially purified from bone.

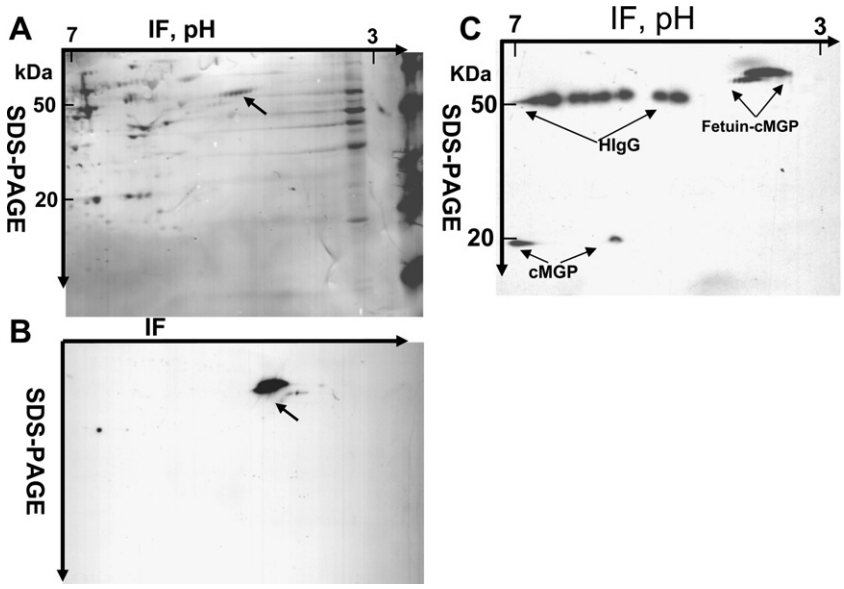

Fig. 2. Presence of fetuin and a fetuin–MGP complex in normal articular chondrocytes. Samples of cell lysates from normal articular chondrocytes were separated by 2-D gel electrophoresis as described in the Materials and methods. (A) Coomassie blue staining and (B) Western blot of the proteins shown in panel A with the mouse monoclonal anti-human fetuin antibodies. The characteristic shape of the stained fetuin is pointed out by an arrow in panel A and panel B shows that stained fetuin protein reacts with the anti-fetuin antibody (arrow). (C) Western blot of fetuin immunoprecipitated with mouse monoclonal fetuin antibodies from an RIPA buffer extract of normal chondrocytes and developed with mouse anti-cMGP antibody. The most heavy protein band labeled fetuin–cMGP shows that the characteristically shaped fetuin molecule (arrows) reacts with the anti-cMGP antibody. The anti-cMGP antibody also recognized free cMGP that was seen on the blot (arrows, cMGP). The heavy chain of the anti-fetuin antibodies used to immunoprecipitate fetuin is recognized by the secondary monoclonal horseradish conjugated goat anti-mouse antibodies (labeled HIgG).

24 h [Fig. 3(D and E)]. Figure 3(F) shows the proteins on the Coomassie blue stained PVDF membrane that was used for Western blotting. Staining of the membrane following Western blotting demonstrates very similar loading of proteins in the OA and normal lanes. We conclude from these experiments that vesicles shed from normal chondrocytes, but not from OA chondrocytes, contain fully γ-carboxylated MGP while both normal and OA vesicles contain fetuin.

To provide evidence that fetuin and cMGP seen on the vesicle blots in (Fig. 3) from normal chondrocytes were assembled as a complex, we repeated the immunoprecipitation experiments, with anti-fetuin antibody, that we had performed with cell extracts. The anti-cMGP antibody again identified the fetuin–MGP complex and also significant amounts of free cMGP (Fig. 4). On the same blot, the heavy chain of the goat anti-IgG used for immunoprecipitation was not recognized by the secondary anti-mouse IgGs antibodies

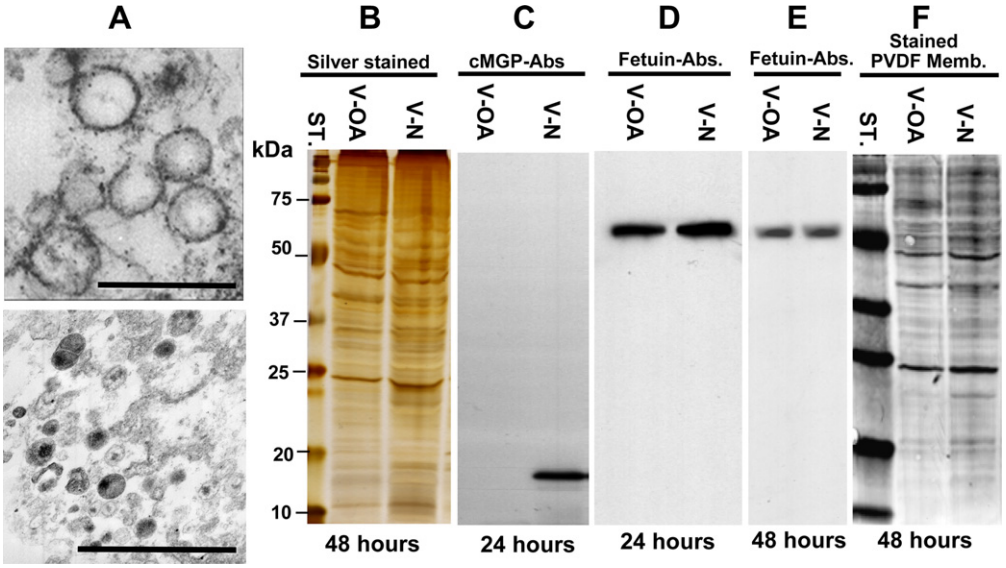

Fig. 3. MGP and fetuin in vesicles from normal and OA chondrocytes. Vesicles from normal (N) and osteoarthritic (OA) serum-free cultures (24 or 48 h serum-free as indicated) were isolated by centrifugation as described in Materials and methods. (A) Electron microscopic images of matrix vesicles isolated from OA chondrocyte cell cultures. The bars represent a distance of 300 nm. Two panels are shown in order to demonstrate the variety of vesicles. (B) Silver stained proteins present in the vesicles (V) from normal (lane V-N) and osteoarthritic (V-OA) cells. Equal amounts of total protein were loaded in each lane. (C) Western blots with the anti-cMGP antibody of the proteins shown in panel B. This conformational specific antibody recognizes only the mature fully γ-carboxylated MGP in the matrix vesicles shed from normal chondrocytes. (D) and (E) Western blots of the proteins in vesicles isolated at 24 and 48 h from cultured cells, respectively, blotted with anti-fetuin antibody. Equal amounts of total proteins were loaded in each lane in panels D and E. (F) Coomassie blue stained proteins on the PVDF membrane used for fetuin Western blotting shown in panel E.

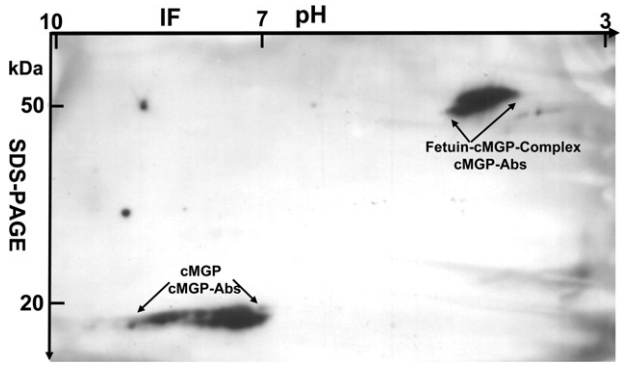

Fig. 4. Presence of the cMGP–fetuin complex in vesicles from normal chondrocytes. An RIPA buffer extract of vesicles isolated from normal chondrocytes was immunoprecipitated with affinity purified goat anti-human fetuin antibody. The immune-precipitated proteins on Sepharose–Protein-A/G beads were separated in 2-D-SDS-PAGE gels and Western blotted with the mouse monoclonal anti-cMGP antibody. The characteristic shape of the fetuin–cMGP complex was seen as the high molecular weight band (arrows) and free cMGP was also seen (low molecular weight band).

used for immunoprecipitation. Since fetuin was found to be present in chondrocytes cultured in serum-containing and serum-free medium, we used immunocytochemistry to examine uncultured cartilage sections for fetuin. Fetuin was seen in chondrocytes present within the cartilage matrix (Fig. 5). Higher magnification images revealed fetuin in particles distributed in the cytosolic part of the cells which may be intra-cellular vesicles [Fig. 5(B)]. Linking this finding to our earlier finding of MGP in intra-cellular vesicles in chondrocytes by electron microscopy[10], adds additional support to our hypothesis that intra-cellular vesicles carry fetuin–cMGP complexes.

Finally, we provide evidence for an uptake mechanism of fetuin by cultured chondrocytes. As shown in (Fig. 6), biotinylated fetuin binds to the cell membrane of chondrocytes at 4°C (panel A) and is taken up by endocytosis to appear intra-cellularly (panel B), when the temperature is raised to 37°C. The mechanism of uptake is still unknown, but the results parallel data from uptake studies of other proteins when the same experimental design was used[26].

Discussion

These results demonstrate an important difference between chondrocytes isolated from normal and OA cartilage that might explain the increased propensity for matrix mineralization in OA cartilage. Although both normal and OA chondrocytes produced the mineralization inhibitory protein MGP, OA cells produced primarily ucMGP while normal cells produced the functional cMGP. This was likely due to reduced levels of γ-carboxylase activity noted in OA cells. In addition, we found that chondrocytes imported the MGP binding protein fetuin by endocytosis and formed a fetuin–MGP complex. Although there was no apparent difference between normal and OA cells in the content of fetuin present in vesicles shed from the cells, only vesicles from normal cells contained the functional γ-carboxylated MGP. Fetuin, by itself, is a Ca^{++} phosphate binding protein and an inhibitor of basic Ca^{++} phosphate crystal formation[16]. Fetuin is synthesized by the liver and secreted into blood[30]. In addition to being an important calcium phosphate carrying protein in blood which prevents precipitation of calcium phosphate salts[20], it is also carried to the bone where it is found as one of the most abundant noncollagenous proteins[18]. In bone, fetuin is a strong binding protein of fully γ-carboxylated MGP which forms a complex with MGP that is carried from bone into blood as a fetuin–MGP complex[15]. Binding of the complex is believed to be mediated through binding *via* calcium phosphate that is attached to the complex. However the serum fetuin–MGP complex is isolated from serum in the presence of 60 mM EDTA[17] which suggest that additional binding interactions between the two proteins also are involved. These studies support our finding of the fetuin–MGP complex in 2-D-SDS-PAGE gels. Price *et al.*[17] propose that the strong calcification inhibitory properties of the fetuin–MGP complex results from the fact that the complex has two protein components with strong calcium phosphate binding capacity.

In cartilage as in bone, the fetuin–MGP complex is likely to bind to nascent mineral nuclei thus preventing the growth of mineral. Basic calcium phosphate crystals appear to be the predominant form present in OA cartilage[3]. We propose that the complex formed between fetuin and cMGP significantly enhances the ability of these proteins to inhibit crystal formation. Fetuin, in vesicles shed from normal chondrocytes, could carry an additional strong calcification inhibitor (cMGP) into the normal cartilage matrix and the fetuin–cMGP complex would be expected to enhance the anti-calcification environment. These results are supported by data published by Reynolds *et al.*[21] on Ca^{++} phosphate crystal formation in cell cultures of VSMCs that also shed vesicles. The authors found that the vitamin K antagonist warfarin, when added to the cell cultures, triggered Ca^{++} phosphate crystal formation. Warfarin inhibits carboxylation and thus formation of cMGP. It is not known if warfarin directly affects the function of fetuin. How insoluble

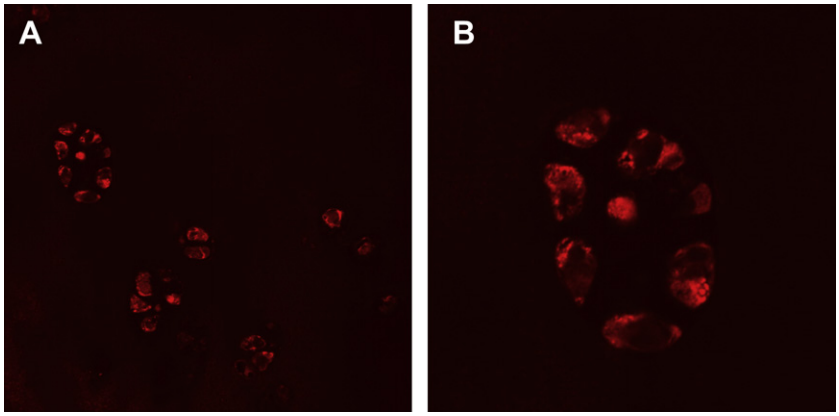

Fig. 5. Immunolocalization of fetuin in human articular cartilage. Sections from human knee articular cartilage were processed for confocal microscopy as described in detail in Materials and methods. The section was reacted with a highly specific monoclonal rat recombinant anti-human fetuin peptide antibody followed by visualization on the immune complexes with a donkey rhodamine conjugated anti-mouse antibody. Panel A shows fetuin to be present in several lacuna. Panel B is an enlarged image to examine intra-cellular details of the rhodamine stained spots.

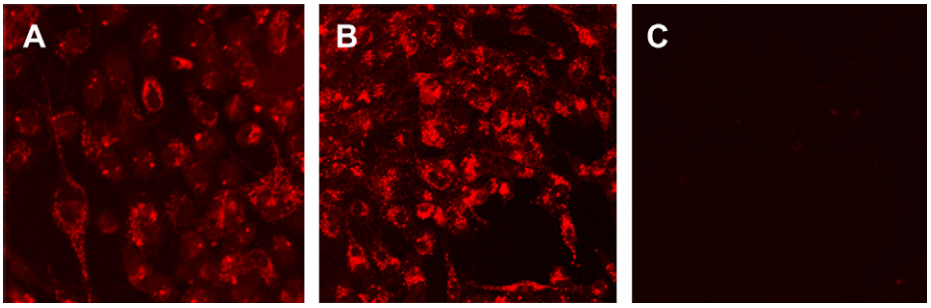

Fig. 6. Biotin-labeled fetuin binding and uptake by cultured human chondrocytes. Human chondrocytes were depleted of endogenous fetuin as described in Materials and methods and incubated at 4 or 37°C in serum-free medium containing biotin-labeled human fetuin. (A) Cells incubated with biotin-labeled human fetuin for 30 min at 4°C. (B) Cells incubated with biotin-labeled fetuin for 30 min at 4°C, followed by incubation at 37°C for an additional 30 min; (C) same experiment as in (B) except that unlabeled fetuin was used in the incubations. Rhodamine epifluorescence images were obtained using a Zeiss Axioskop equipped with a digital camera and Axovision imaging software as described in Materials and methods.

cMGP leaves viable cells has never been understood in great detail. Our results present new data consistent with a cellular mechanism for the release of MGP. We present evidence for the existence of an intra-cellular fetuin–cMGP complex in normal chondrocytes that could serve as a chaperone mechanism needed to carry the insoluble cMGP packed intra-cellularly in vesicles which are shed into the extracellular matrix by exit of these vesicles from the cell membrane. Previously, we had noted the presence of MGP in vesicle like structures within chondrocytes and in the immediate matrix[10]. We propose that cMGP, but not ucMGP binds to Ca^{++} phosphate carrying intra-cellular fetuin and becomes embedded in lipid covered vesicles possibly originating from the Golgi apparatus membrane, a mechanism known to be involved in carrying a variety of other proteins intra-cellularly[31]. Because we found the complex in vesicles shed from normal, non-mineralizing, articular cartilage and because we did not assess the mineralizing activity of these vesicles, we did not refer to them as authentic matrix vesicles but rather simply as “vesicles”.

Most studies on soft tissue calcification have been carried out on the arterial vessel wall[32] and blood borne fetuin has been shown to be endocytosed by VSMCs in culture[20]. In this paper, we show for the first time that cartilage, a non-vascularized tissue, contains fetuin. Results from experiments using confocal microscopy of human cartilage tissue supported the cell culture data and provide evidence to suggest that chondrocyte fetuin uptake is an ongoing *in vivo* mechanism. Furthermore, we showed fetuin uptake by chondrocytes in cell culture by a mechanism which was found to be similar to the mechanism of fetuin uptake by cultured VSMCs. Based on our data and data published by other investigators[20,21] it appears that the mechanism of action of fetuin and cMGP at the cellular level in various cells share many biochemical and cellular similarities. Recent studies have suggested a link between low vitamin K intake and mineralization in the blood vessel wall. The majority of the work to date suggests that MGP is a primary regulator of blood vessel mineralization and that increased blood vessel mineralization may occur when MGP is not fully carboxylated[6,33–35]. Further studies are indicated to determine if similar mechanisms are at play in articular cartilage. Our results and previous studies showing increased cartilage mineralization in MGP knock-out mice[7], and inhibition of cartilage mineralization by MGP overexpression[36] are consistent with an important regulatory role for MGP in controlling matrix mineralization in cartilage. Keutel Syndrome, which is characterized by abnormal mineralization in multiple cartilages including the growth plate[37], has been found to be associated with mutations in MGP[38]. Although articular cartilage mineralization *per se* has not been described in Keutel Syndrome, the vast majority of the reports have been in children and it is possible that abnormal articular cartilage mineralization would not appear until more advanced age. It is also possible that other mineralization inhibitors can compensate for a congenital lack of MGP in articular cartilage or that MGP is more important in inhibiting pathological mineralization associated with OA. Additional studies are needed to demonstrate the ability of the fetuin–cMGP complex to inhibit mineralization induced by adult articular chondrocytes and to determine if vitamin K supplementation might be useful for preventing abnormal cartilage mineralization in older adults.

Author contributions

All three authors contributed to the conception and design of the study, analysis and interpretation of data, drafting and revising the manuscript for intellectual content, and final approval of the version submitted. RW and RL also contributed to acquisition of data and LS to provision of study materials. All three authors take responsibility for the integrity of the work.

Conflict of interest

None of the authors have financial or personal relationships with people or organizations that could inappropriately influence this work.

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